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# Transforming growth factor β stimulates mammary adenocarcinoma cell invasion and metastatic potential

**(13762NF mammary adenocarcinoma/type IV collagenase/gelatinase/heparanase)**

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ABSTRACT **The experimental metastatic potential of 13762NF mammary adenocarcinoma clone MTLn3 was tested after pretreatment in serum-free medium containing transforming growth factor (TGF) $\beta_1$ at 0–5000 pg/ml. Lung colonies were measured 2 weeks after inoculation in syngeneic F344 rats, and a bell-shaped dose–response curve with 2- to 3-fold increase in number of surface lung metastases was seen. Maximal enhancement occurred at the 50 pg/ml dose level. The effect was specific because addition of neutralizing anti-TGF-β antibody blocked the stimulatory activity at all levels of TGF-$\beta_1$ pretreatment, but when antibody was given alone, neutralizing anti-TGF-β antibody had no effect on untreated cells. Increased metastatic potential appears to be from an increased propensity of cells to extravasate as tested in the membrane invasion culture system. MTLn3 cells penetrated reconstituted basement-membrane barriers 2- to 3.5-fold more than did untreated control cells, depending upon length of TGF-$\beta_1$ exposure. Increased invasive potential is apparently due, in part, to a 2- to 6-fold increase in type IV collagenolytic (gelatinolytic) and a 2.4-fold increase in heparanase activity. TGF-$\beta_1$ treatment of MTLn3 cells did not alter their growth rate or morphology in the presence of serum; however, growth was inhibited in serum-free medium. Likewise, adhesion to human umbilical vein endothelial cell monolayers or to immobilized reconstituted basement membrane or fibronectin matrices was unchanged. These results suggest that TGF-$\beta_1$ may modulate metastatic potential of mammary tumor cells by controlling their ability to break down and penetrate basement-membrane barriers.**

Transforming growth factor (TGF) β is a 25-kDa disulfide-linked polypeptide dimer that elicits numerous changes in cellular behavior, including differentiation of epithelial cells (for reviews, see refs. 1–4), modifying proliferation in a wide variety of cell types (1, 4–10), inhibition of angiogenesis (11, 12), deposition of extracellular matrix components (11–15), alteration of basement-membrane-degrading enzyme production and/secretion (refs. 11, 16–19; B. Korczak, R. S. Kerbel, and J. Dennis, personal communication), and changes in cellular-adhesive properties (12, 14, 15). The ability of TGF-β to modulate cell growth and cell–matrix interactions suggests a role of this polypeptide class in modifying the invasive and metastatic potential of tumor cells.

Recent data have shown that tumor cells secrete factors that modulate the metastatic potentials of other tumor cells directly (20–24) or indirectly through intermediary cells (25–28). However, the nature of the metastasis-modifying signals remains unknown. TGF-β is associated, to varying degrees, with processes involved in the metastatic cascade. For example, TGF-β mRNA levels are elevated in malignant human breast-tumor biopsy specimens (29); TGF-β is strongly growth inhibitory to human MCF7 breast carcinoma cells (8–10); and TGF-β is secreted by a variety of cell types often associated with metastasizing tumor cells (for reviews, see refs. 1–4). Therefore, we investigated the regulatory role of TGF-β on tumor cell invasive and metastatic potentials. The results presented here provide evidence that brief exposure to TGF-β results in enhanced ability to penetrate a basement-membrane-like matrix *in vitro* and to form lung colonies when injected *in vivo*.

## MATERIALS AND METHODS

**Cell Lines and Tissue Culture.** 13762NF rat mammary adenocarcinoma clone MTLn3 cells were grown and maintained as described (28, 30). Briefly, cells were grown in α-modified Eagle's minimal essential medium (α-MEM) supplemented with 5% fetal bovine serum (FBS) in 100-mm tissue culture dishes (Corning) without antibiotics. Cells were subcultured when the plates became 70–80% confluent by using 0.25% trypsin solution in $Ca^{2+}$- and $Mg^{2+}$-free Dulbecco's phosphate-buffered saline. All cultures were routinely tested and found negative for *Mycoplasma* sp. contamination.

**Animals.** Pathogen- and virus-free, 6- to 7-week old female Fischer 344/NHSd (F344) rats were obtained from Harlan Sprague–Dawley. Animals were maintained under the guidelines of Glaxo Research Laboratories and the National Institutes of Health. All protocols were approved by Glaxo's Institutional Animal Care and Use Committee. Rats were fed Purina rodent chow and tap water (chlorine content was <5 ppm) ad libitum.

**Treatment with Growth Factors.** Subconfluent cultures were seeded at least 48 hr before use onto 100-mm tissue culture dishes. At desired confluence (50%), the medium was removed, the cells were washed with prewarmed serum-free α-MEM, and medium was replaced with serum-free α-MEM containing the appropriate concentration of growth factor, carrier, antibody, or combinations of the above. Cells were treated for 4 hr before detachment and use in the experimental assays.

**Reagents.** Platelet-derived human or porcine TGF-$\beta_1$, porcine TGF-$\beta_2$, human platelet-derived growth factor, and TGF-β neutralizing antibody (rabbit anti-swine) were obtained from R&D Systems (Minneapolis). TGF-β was reconstituted to 1 μg/ml in 4 mM HCl and 1% bovine serum albumin-fraction V (wt/vol) in water. The stock solution was diluted 1:100 in α-MEM and added to the cells to achieve the final concentrations indicated. TGF-β neutralizing antibody was reconstituted in phosphate-buffered saline, and sufficient antibody solution was added to neutralize activity of the maximum concentration of TGF-β used in each experiment

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Abbreviations: TGF, transforming growth factor; MICS, membrane invasion culture system; RBM, reconstituted basement membrane; α-MEM, α-modified Eagle's minimal essential medium; FBS, fetal bovine serum.

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(40 μg of IgG completely neutralized activity of TGF-β at 1 ng/ml). Insulin-like growth factor I (somatomedin C) and NuSerum were purchased from Collaborative Research, and human recombinant TGF-α was from GIBCO. α-MEM, phosphate-buffered saline, Dulbecco's modified Eagle's medium (DMEM)/Ham's (F-12) (1:1, vol/vol), $Ca^{2+}$- and $Mg^{2+}$-free Dulbecco's phosphate-buffered saline, and Hanks' balanced salt solution were purchased from Irvine Scientific, and FBS was supplied by Biocell (Carson, CA). All other reagents were purchased from Sigma.

**Experimental Metastasis Assay.** Subconfluent MTLn3 cells were detached by removal of medium followed by addition of 1 ml of ice-cold 0.25% trypsin solution. The enzyme solution was aspirated and replaced by another 1 ml of ice-cold 0.25% buffered trypsin solution, and the plates were placed into an incubator. When the cells began to retract and detach, the plates were gently tapped to dislodge the remaining cells, and cold α-MEM containing FBS was added to inactivate the trypsin. The cells were pelleted by centrifugation, the medium was removed, and the cells were resuspended in ice-cold Hanks' balanced salt solution at $5 \times 10^5$ cells per ml. Cells were collected in polypropylene centrifuge tubes and placed into an ice bath. Cells were counted by using a hemacytometer, and viability was determined by trypan blue dye exclusion. Only cell suspensions with viabilities >95% were used for inoculation. Animals received $1 \times 10^5$ cells i.v. into the lateral tail vein. Two weeks postinoculation, the animals were killed by using Metofane [methoxyflurane, Pitman-Moore (Washington Crossing, NJ)] anesthesia and subjected to complete gross necropsies. Lungs were fixed in a neutral-buffered formalin/Bouins' fixative solution, 5:1 (vol/vol). The number of surface lung metastases was counted with the aid of a dissecting microscope as described (28, 30, 31).

***In Vitro* Tumor Cell Invasion Assay.** To measure tumor cell invasion, the membrane invasion culture system (MICS) (32, 33) was used with minor modifications. Instead of the 12/15 well configuration used in the original MICS, we used a 24-well manifold chamber fitted with a silicon gasket between the upper and lower plates to ensure no lateral cross-contamination between wells (Neuroprobe, Cabin John, MD). Then, sterile 10-μm polycarbonate filters (Nucleopore) were coated with a reconstituted basement-membrane (RBM) barrier, Matrigel (Collaborative Research), and dried under laminar flow. The lower chambers of MICS were filled with 5% NuSerum-supplemented α-MEM. NuSerum was used because it contains lower levels of most serum proteinase inhibitors (33). The coated filters were cut and then placed between the upper and lower plates of the invasion chamber, and 1.5 ml of 5% NuSerum-supplemented α-MEM containing $1 \times 10^5$ tumor cells was placed into the upper wells. After 72 hr, the medium from the upper wells was removed and discarded, the medium from the lower wells was removed and stored in polypropylene centrifuge tubes, and 0.125% trypsin/2 mM EDTA solution in $Ca^{2+}$- and $Mg^{2+}$-free Dulbecco's phosphate-buffered saline was added to the lower wells and incubated to remove the tumor cells that had invaded into the lower wells. The cells were removed by means of sampling ports and added to the corresponding tubes of medium from each well. Cells were separated from the medium/trypsin/EDTA by filtration through a 0.45-μm-pore polycarbonate filter (Nucleopore) with a Minifold-1 apparatus (Schleicher & Schuell). Trapped cells were fixed, stained, and placed onto a slide, layered with immersion oil, and mounted with a coverslip. Individual tumor cells were counted from replicate samples by using a 20× planapochromat objective on a Nikon Optiphot microscope, and the number of invading cells was compared with simultaneously run, untreated, control cells. Total number of invading cells was calculated based upon the surface area per high-power field and the surface area of the Minifold-well. Percent invasion was then calculated as described (32).

**Type IV Collagenase Assay.** Type IV collagenolytic activity was measured by using N-[$^3$H]acetylated type IV collagen purified from Engelbreth–Holm–Swarm tumor as described (34–36). MTLn3 cells ($5 \times 10^4$) were suspended in 500 μl of serum-free DMEM/F-12, placed on dried N-[$^3$H]acetylated type IV collagen film (20 μg, 15,000 cpm), and TGF-$\beta_1$ at 0–1000 pg/ml was added. Type IV collagenolysis over a 24-hr incubation at 37°C was measured by determining radioactivity in an aliquot of supernatant. $^3$H-labeled degradation products, which were not precipitable in 5% (vol/vol) trichloroacetic acid and 0.1% tannic acid, were measured by liquid scintillation counting.

**Heparanase Assay.** The heparanase assay was done as described (35, 37). Heparan sulfate derivatives labeled with $^3$H (1200 cpm per μg) covalently linked to amino-reactive agarose beads were mixed with a tumor-cell extract (from $2 \times 10^5$ cells treated with TGF-$\beta_1$ at 0–500 pg/ml for 24 hr) in 0.2% Triton X-100/0.2 M sodium acetate, pH 5.0. After 3-hr incubation at 37°C, the reaction mixture was heated at 100°C for 10 min and centrifuged at 18,000 × *g* for 5 min; then radioactivity in an aliquot of the supernatant was measured. Relative heparanase activity is a ratio of TGF-$\beta_1$-treated cell heparanase activity to heparanase activity in control, untreated cells.

**Zymography.** Identification of type IV collagenolytic enzymes secreted by TGF-$\beta_1$-treated MTLn3 cells was performed by electrophoresis of serum-free conditioned medium (DMEM/F-12) in gelatin-embedded polyacrylamide gels followed by incubation and Coomassie blue staining (34, 35). Gelatin was dissolved in 2% $NaDodSO_4$ and then copolymerized with 7.5% acrylamide. Electrophoresis was performed according to the method of Laemmli (38) by using a Protean II dual slab system (Bio-Rad). After electrophoresis, gels were rinsed twice in 2.5% Triton X-100/50 mM Tris·HCl, pH 7.5, and incubated at 37°C for 16 hr in 0.15 M NaCl/10 mM $CaCl_2$/50 mM Tris·HCl, pH 7.5/0.05% $NaN_3$. Gels were stained with 0.005% Coomassie blue and destained with 10% (vol/vol) acetic acid and 10% (vol/vol) isopropanol. Type IV-collagenolytic enzymes were detected as transparent bands on slab gels.

**Statistical Analyses.** Statistical analyses were done using the one-way analysis of variance and Duncan's multiple range test for difference between the means. All experiments were performed at least twice. Animal experiments contained 10–25 rats per group, and MICS experiments were in quadruplicate replicates.

## RESULTS

Pretreatment of subconfluent 13762NF mammary adenocarcinoma clone MTLn3 cells for 4 hr with TGF-$\beta_1$ caused the number of experimental lung metastases to rise 1.5- to 3-fold in a bell-shaped dose–response curve with maximal enhancement occurring at the 50 pg/ml (2 pM) dose (Fig. 1). TGF-$\beta_2$ pretreatment resulted in a similar increase in experimental metastasis formation with similar dose–response. The metastasis-stimulatory effect was specific to the TGF-β family of growth factors because pretreatment with TGF-α (0–5000 pg/ml) induced no change in MTLn3 metastatic potential (Table 1). Likewise, insulin-like growth factor 1 (1.0–100 ng/ml for 4 hr) and platelet-derived growth factor (0.01–10 ng/ml for 4 hr) did not alter experimental metastatic potential of clone MTLn3 (data not shown).

To assure that stimulation of experimental metastatic potential was from TGF-β and not a minor contaminant in the medium or an artifact of acid exposure (used to convert the TGF-β from the latent to the active form), a TGF-β neutralizing antibody was added as TGF-β was added to cells. Incubation of cells with anti-TGF-β alone caused no change in experimental metastasis formation. When anti-TGF-β was

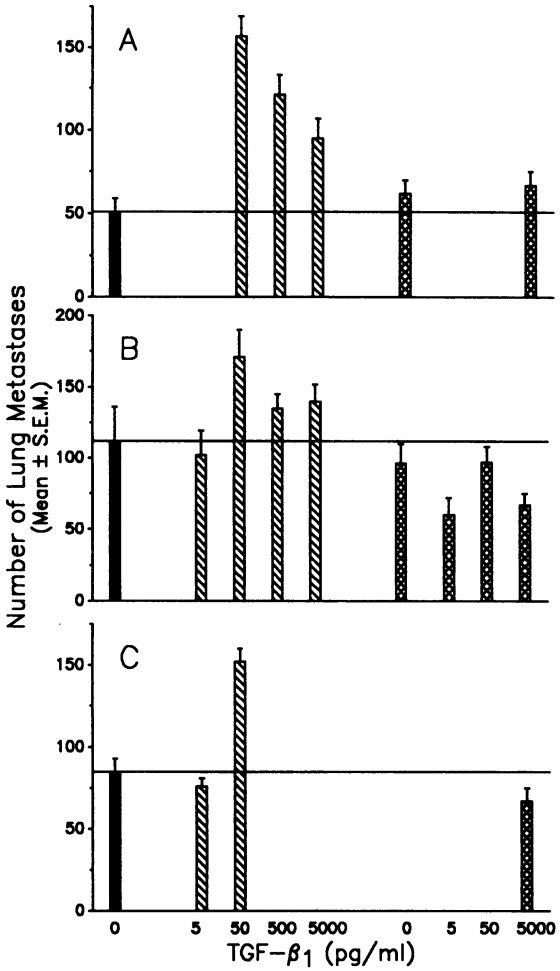


FIG. 1. Experimental metastatic potential of 13762NF mammary adenocarcinoma clone MTLn3: effect of TGF-$\beta_1$. *A*, *B*, and *C* represent independent experiments. Viable tumor cells ($10^5$ per 0.2 ml) were injected into the lateral tail veins of syngeneic F344/NHSd female rats. Animals were killed 14 days postinoculation, lungs were removed, and the numbers of surface lung metastases were counted. ■, Control, untreated cells; ▨, cells pretreated for 4 hr with TGF-$\beta_1$; ▩, cells coincubated for 4 hr with TGF-$\beta_1$ and neutralizing anti-TGF-$\beta$ antibody (40 $\mu$g of IgG per ng of TGF-$\beta$). TGF-$\beta_1$-treated cells at the 50 pg/ml dose differ significantly from control ($P < 0.05$). Error bars = SEM.

added concomitantly with TGF-$\beta$ to MTLn3 cells, the number of lung colonies was similar to controls, indicating that the metastasis-enhancing factor was, indeed, TGF-$\beta$ (Fig. 1).

Table 1. Effect of TGF-$\beta_2$ and TGF-$\alpha$ on stimulation of MTLn3 cell metastatic potential

| Treatment | TGF, pg/ml | Surface lung metastases,* no. | Relative metastatic potential |
|---|---|---|---|
| Control | | 85 ± 8 | 1.00 |
| TGF-$\beta_2$ | 50 | 124 ± 18 | 1.46† |
| | 500 | 104 ± 6 | 1.22 |
| TGF-$\alpha$ | 5 | 94 ± 8 | 1.11 |
| | 500 | 90 ± 7 | 1.06 |
| | 5000 | 108 ± 10 | 1.27 |

*Viable tumor cells ($10^5$ per 0.2 ml) were injected into the lateral tail veins of syngeneic F344/NHSd female rats. Animals were killed 14 days postinoculation, lungs were removed, and the number of surface lung metastases was counted through a dissecting microscope (32). Data are reported as mean ± SEM.
†Statistically different from control ($P < 0.05$).

Likewise, acid carrier had no effect on experimental metastatic potential.

Because TGF-$\beta$ treatment can upregulate cell-surface integrin expression (14, 16, 39) and because cell adhesion has been correlated with metastatic potential (for review, see ref. 40), the ability of TGF-$\beta$-pretreated MTLn3 cells to attach to HUVEC monolayers or to RBM- or fibronectin-coated tissue culture dishes was tested. [$^{125}$I]iododeoxyuridine-labeled tumor cells were added to cell wells containing confluent monolayers of HUVEC cells, coated with RBM or fibronectin, and cell adhesion was determined as described (32). The kinetics and percent maximal adhesion were identical for control and TGF-$\beta$-treated cells (data not shown).

TGF-$\beta$ exerts profound changes in growth properties and differentiation of mammary cells (7–10). Therefore, we determined whether TGF-$\beta$ pretreatment changed metastatic potential by altering MTLn3 cell proliferative capacity. Continuous exposure of MTLn3 cells to TGF-$\beta_1$ or TGF-$\beta_2$ caused a slight, but statistically insignificant, decrease in the growth potential of cells grown in 0.25% FBS-supplemented $\alpha$-MEM as measured by cell counts after 5 days in tissue culture; whereas TGF-$\beta_1$ was potently cytostatic to MTLn3 cells when no FBS was added to the culture medium. When plated cells were grown in serum-free $\alpha$-MEM containing TGF-$\beta_1$ at 5–500 pg/ml for 72 hr, cell number remained constant. In contrast, cell number doubled when grown in serum-free and TGF-$\beta_1$-free $\alpha$-MEM.

In a variety of cell types, TGF-$\beta_1$ or TGF-$\beta_2$ regulates deposition and breakdown of extracellular matrices, including basement membranes (11–20). For tumor cells to form secondary tumors, they must traverse the subendothelial basement membrane surrounding microvessels (32, 36, 40, 41). Together, these observations suggest that TGF-$\beta$ may modulate metastasis by altering the ability of the tumor cells to penetrate basement membranes. To test this hypothesis, we modified the MICS (32, 33) in which tumor-cell-invasion potential across RBM can be quantified. Pretreatment of MTLn3 cells for 4 hr with TGF-$\beta_1$ at 50 pg/ml increased the relative invasive potential 1.72- to 2.12-fold (Table 2). Pretreatment on tissue culture dishes, followed by continuous exposure to TGF-$\beta_1$ at 50 pg/ml while in the MICS chamber, stimulated invasion 2.74- to 3.56-fold, indicating that continued TGF-$\beta_1$ exposure further stimulated mammary-tumor-cell invasion. Continuous exposure to TGF-$\beta_1$ in MICS only (without pretreatment) enhanced metastatic potential 2.06- to 3.02-fold, remarkably similar to the 4-hr pretreatment group.

Type IV collagenolytic activity of 13762NF rat mammary adenocarcinoma cell clone MTLn3 was measured as described (35) by using N-[$^3$H]acetylated type IV collagen. MTLn3 cells ($5 \times 10^4$) suspended in 500 $\mu$l of serum-free $\alpha$-MEM were placed on a dried N-[$^3$H]acetylated type IV collagen film (20 $\mu$g, 15,000 cpm), and type IV collagenolysis over a period of 24-hr incubation was measured (Fig. 2). As with the *in vivo* experimental metastasis assay and the *in vitro* MICS assay, a bell-shaped dose–response was obtained with maximal 6-fold stimulation at the 50 pg/ml dose of TGF-$\beta_1$. Identification of the type IV collagenolytic enzymes secreted by TGF-$\beta_1$-treated MTLn3 cells was performed by electrophoresis of serum-free conditioned medium in a gelatin-embedded polyacrylamide followed by incubation and Coomassie blue staining, as described (35, 36). TGF-$\beta_1$ effects were relatively specific for the 92-kDa and 64-kDa bands, suggesting some differential gene regulation by TGF-$\beta$ (Fig. 3). The 92-kDa and 64-kDa enzymes were enhanced by TGF-$\beta_1$ exposure at 50 and 1000 pg/ml for 24 hr. At 72 hr, the 92-kDa and 64-kDa activities were significantly more elevated in the 5 and 50 pg/ml-treated cells, whereas the 64-kDa and 92-kDa activities returned to near control levels in the 1000 pg/ml-treated cells. This result could also be due, in

Table 2. Pretreatment of 13762NF mammary adenocarcinoma cell clone MTLn3 with TGF-$\beta_1$ stimulates invasion through reconstituted basement-membrane barriers in MICS

| Experiment | Group* | Invasion, % | Relative invasive potential |
|---|---|---|---|
| 1 | 1 | 0.18 ± 0.02 | 1.00 |
| | 2 | 0.31 ± 0.04 | 1.72† |
| | 3 | 0.64 ± 0.05 | 3.56† |
| | 4 | 0.37 ± 0.04 | 2.06† |
| 2 | 1 | 0.42 ± 0.06 | 1.00 |
| | 2 | 0.89 ± 0.10 | 2.12† |
| | 3 | 1.15 ± 0.12 | 2.74† |
| | 4 | 1.27 ± 0.14 | 3.02† |

Tumor cells were seeded at $10^5$ (in 1.5 ml) per well in the MICS chamber. Chambers were incubated for 72 hr, medium containing invading cells was removed through sampling ports, and cells were isolated by filtering them onto a 0.45-$\mu$m filter with a Minifold filtration apparatus. Retained cells were fixed, stained, and counted as described (33). Relative invasive potential was calculated as the ratio of treated vs. untreated tumor cells within the same experiment ($n$ = 6).

*Group 1, control (no treatment); group 2, pretreatment with TGF-$\beta_1$ at 50 pg/ml for 4 hr; group 3, pretreatment with TGF-$\beta_1$ at 50 pg/ml for 4 hr plus TGF-$\beta_1$ at 50 pg/ml in MICS; group 4, TGF-$\beta_1$ at 50 pg/ml in MICS.

†$P < 0.01$ from control by one-way analysis of variance and Duncan's multiple range test.

part, to the cytostatic effects of TGF-$\beta_1$ on MTLn3 cells in serum-free medium.

The ability of malignant tumor cells to break down another major component of vessel intimal layers, heparan sulfate proteoglycan, was measured as described (36, 37). The relative heparanase activity was determined by calculating the ratio between TGF-$\beta_1$-treated cells and simultaneously tested,

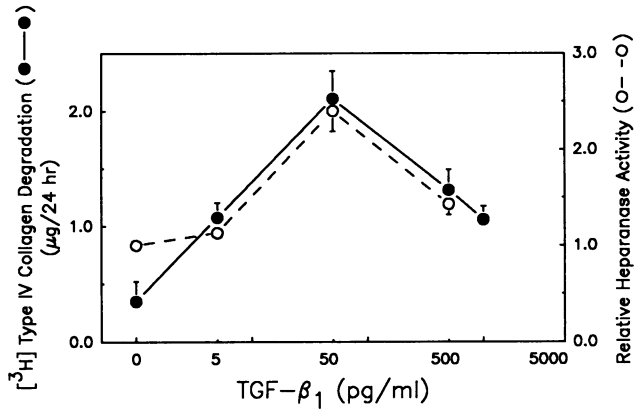


FIG. 2. Stimulation of type IV collagenolytic activity (●) and heparanase activity (○) from MTLn3 cells treated for 24 hr with TGF-$\beta_1$. Type IV collagenolytic activity of 13762NF mammary adenocarcinoma cell clone MTLn3 was measured by published methods (35) with N-[$^3$H]acetylated type IV collagen. MTLn3 cells ($5 \times 10^4$) suspended in 500 $\mu$l of serum-free DMEM/F-12 were placed on a dried N-[$^3$H]acetylated type IV collagen film (20 $\mu$g, 15,000 cpm), TGF-$\beta_1$ was added, and type IV collagenolysis over a 24-hr incubation was measured. Heparanase activity was determined as described (35, 37). Heparan sulfate derivatives labeled with $^3$H (1,200 cpm/$\mu$g) covalently linked to amino-reactive agarose beads were mixed with a tumor-cell extract ($2 \times 10^5$ cells) in 0.2% Triton X-100/0.2 M sodium acetate, pH 5.0. After 3-hr incubation at 37°C, the reaction mixture was heated at 100°C for 10 min and centrifuged at 18,000 × $g$ for 5 min; then radioactivity in an aliquot of the supernatant was measured. Relative heparanase activity = TGF-$\beta_1$-treated cells per control cells (×100). Data points indicate mean ± SD.

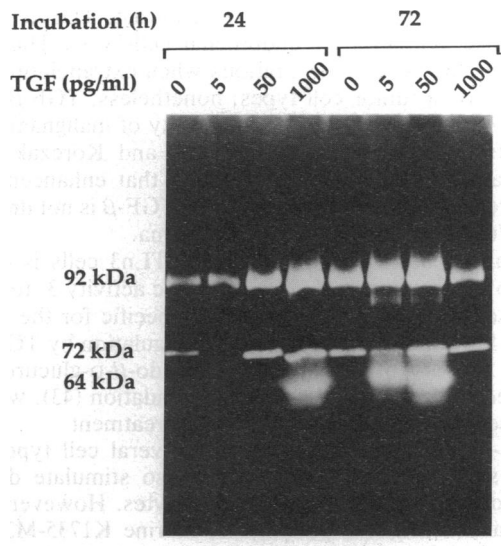


FIG. 3. Identification of TGF-$\beta$-induced type IV collagenolytic proteinases. MTLn3 cells were cultured in the presence of TGF-$\beta_1$ at $5 \times 10^5$ cells per ml in serum-free DMEM/F-12, 1:1 (vol/vol), for 24 hr or 72 hr at 37°C, and 2-ml aliquots of conditioned medium were concentrated and immediately analyzed by electrophoresis in gelatin-embedded polyacrylamide gels followed by 16-hr incubation and Coomassie blue staining for detection of *in situ* type IV collagenolytic activity.

untreated control cells (Fig. 2). Heparanase activity increased 2.4-fold in MTLn3 cells treated with TGF-$\beta_1$ at 50 pg/ml.

## DISCUSSION

The most lethal attribute of cancer is the ability of specialized tumor cells to spread to distant sites and form secondary tumors. To metastasize, a tumor cell must invade and enter the vasculature, survive transport in the circulation, bind to and traverse the vascular endothelium and basement membrane, and divide in a foreign location (40, 41). Only specialized subpopulations of tumor cells can perform all of the steps necessary in this complex, highly selective cascade (40, 41). Yet, recent data from several laboratories suggest that signals from the environment can modulate the metastatic potentials of individual cells (20–28).

TGFs elicit behavioral changes in a cell-dependent manner. Some changes include rates of extracellular matrix deposition, assembly, and breakdown (11–18, 42); expression of adhesion receptors (12, 14, 15); chemotaxis and chemokinesis (12, 14, 42); immune cell activation and/or inhibition including phagocytosis (1–4); and growth potential (1–4, 8–12). Several of these phenotypes are important to the ability of a tumor cell to successfully form a secondary tumor. Therefore, we wanted to determine whether TGF-$\beta$ treatment of tumor cells could regulate, in part, the metastatic phenotype.

Our results show that pretreatment of 13762NF mammary adenocarcinoma cells with TGF-$\beta_1$ or TGF-$\beta_2$ stimulates lung colonization potential ≈1.5- to 3-fold. The enhanced metastasizing potential is partially due to TGF-$\beta$-induced type IV collagenolytic (gelatinolytic) and heparanase activities that result in increased ability of MTLn3 cells to degrade and traverse RBM barriers. This finding confirms those of Akedo and colleagues (19), who found stimulation of ascites hepatoma-cell invasion through fibroblast monolayers *in vitro*, and D. L. Mooradian, J. B. McCarthy, G. Spencer, and L. T. Furcht (personal communication) who showed enhanced invasion of K1735 melanoma clones into type I collagen gels. Results with the 13762NF mammary adenocarcinoma are consistent with TGF-$\beta_1$-stimulated collagenase (ref. 42 and Korczak *et al.*, personal communication) and urokinase (17)

secretion by other cell types. In contrast, TGF-$\beta$ inhibits proteinase synthesis by endothelial cells (11). This result reiterates the need to be cautious when extrapolating these findings to all tumor cell types; nonetheless, TGF-$\beta$ stimulates collagenolytic activity in a variety of malignant histiotypes from several species (ref. 17; and Korczak *et al.*, personal communication), suggesting that enhancement of invasive and metastatic potentials by TGF-$\beta$ is not unique to the 13762NF mammary adenocarcinoma.

Enhanced invasive potential by MTLn3 cells is due, in part, to TGF-$\beta_1$ increasing gelatinolytic activity 3- to 6-fold. Because the effects were relatively specific for the 92-kDa and 64-kDa bands, differential gene regulation by TGF-$\beta_1$ is likely. Activity of heparanase, an endo-$\beta$-D-glucuronidase involved in basement-membrane degradation (43), was also increased 1.5- to 2.5-fold by TGF-$\beta_1$ treatment.

TGF-$\beta$ is a chemoattractant for several cell types (12), which suggests that TGF-$\beta$ could also stimulate directed tumor-cell migration or recruit leukocytes. However, TGF-$\beta_1$-enhanced invasive potential of murine K1735-M2 melanoma cells is not due to induced chemotaxis (Korczak *et al.*, personal communication). Since invasion in MICS takes at least 48 hr, a chemotactic gradient could not be maintained to conclusively evaluate the role of chemotaxis in our system. *In vivo* it is more likely that TGF-$\beta$ might recruit leukocytes (i.e., polymorphonuclear leukocytes, macrophages), which are known to assist tumor-cell extravasation (25, 28, 36).

Because TGF-$\beta$ can elevate the transcription and number of cell-adhesion receptors expressed in a variety of cells, metastasis stimulation could result from increased adhesion. However, TGF-$\beta_1$-pretreated MTLn3 cells exhibited no difference in rate of, or maximal levels of, adhesion to RBM or fibronectin matrices or endothelial monolayers (data not shown).

The results presented here clearly show that exposure of tumor cells to exogenous growth factors can stimulate *in vitro* tumor cell invasion and *in vivo* metastatic potential. At present, we can only speculate the most likely sources of TGF-$\beta$ *in vivo*. While not ubiquitous, TGF-$\beta$ is secreted by many cell types—including platelets, macrophages, lymphocytes, endothelial cells, and some tumor cells—which are, to varying degrees, involved in metastasis. Immunohistochemical analysis of cryostat-sectioned MTLn3 primary tumors reveals a light membranous staining with anti-TGF-$\beta$ antibodies (data not shown). This staining shows that some of the TGF-$\beta$ could, indeed, be coming from the mammary tumor cells themselves. However, TGF-$\beta$ need not necessarily be an autocrine factor, although it may be in some tumors.

Tumor invasion and metastasis are complex, multistep phenomena. It is highly unlikely that a single molecule will regulate these phenotypes. Nonetheless, because TGF-$\beta$ is produced by a plethora of normal and abnormal cell types associated with various stages of the metastatic cascade, it will probably be in the extracellular milieu at strategic times to influence malignant properties. TGF-$\beta$ and other, as yet uncharacterized, factors may serve to regulate the metastatic phenotype by overcoming subthreshold deficiencies in some cells and inhibiting biochemical processes in others. Characterization of these factors should allow identification of targets for developing therapeutics useful in the treatment of malignant cancer.

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